**Introduction**

MilliporeSigma Simplicon™ Technology can be used to introduce multiple genes into cells with transfection via a single, synthetic, polycistronic, self-replicating RNA vector. The transfected cell line can be placed under selection to maintain expression of the proteins of interest for a limited number of cell divisions, while leaving no genetic footprint in the modified cell line. Recently, this technology was successfully utilized to introduce four, integration-free, reprogramming factors into human iPSCs.1 For this work, we created three Simplicons™ vectors expressing Phase I, human, xenobiotic metabolizing enzymes. The first vector expressed just a single cytochrome P450 enzyme (CYP3A4), to determine optimal expression conditions and activity profile in HepG2 cells and HEK293 cell lines which stably expressed human liver transporters. To perform this optimization, testosterone was used a substrate to measure 6 beta-hydroxysteroid metabolites produced via mass spectrometry. Subsequently, we replaced the IPSC reprogramming factors with five cytochrome P450 enzymes (e.g. CYP3A4A, CYP2C9, CYP2E1, CYP1A2 and CYP2D6) in two differently designed vectors and tested each separately in a 96-well plate assay using the same cell lines. Activity of each enzyme was quantified by mass spectrometry of the metabolites produced from the substrates testosterone, tolbutamide, chlordiazepoxide, phenacetin and buproprion. Relative expression of each enzyme was determined by qPCR measurement. Unlike the HepG2 cell line, to achieve substantial uptake, HEK293 cells were transfected to stably express liver transporters (i.e. OATP1B1 or OATP1B3) while cytochrome P450 metabolites varied with the substrate used, both cell lines show a 10-fold increase of testosterone metabolism over background in a 96-well plate format, indicating hours of substrate incubation. Cell lines transfected with a Simplicon™ vector, scaled, frozen and subsequently assayed (6 days, total), demonstrated a similar metabolic profile to those assays assayed post-transfection. Currently, most commercial high throughput cell based assay systems use gold clean sets, which lack metabolic competence. Here, the MilliporeSigma Simplicon™ Technology is used to create a cell line which expresses and stimulates metabolic activity of five human xenobiotic metabolizing enzymes, and can be scaled to screen thousands of chemical compounds in a 96-well plate format.

**Methods**

**Simplicon Design:** Two larger RNA constructs were created, each containing five CYP450 enzymes (Plan 2 and Plan 3) separated by either a 2A peptide cleavage site, or an IRES sequence. Additionally, a smaller construct containing only the CYP3A4 enzyme was designed to begin experiments while the larger constructs were being assembled. A puromycin resistance gene was included on all constructs.

**Results**

Figure 1 graphically shows the relative amount of mRNA of each of the CYP450 enzymes in cells transfected with the Plan 3 Simplicon™ design. While cytochrome P450 metabolites varied with the substrate used, both cell lines show a 10-fold increase in testosterone metabolism over background in a 96-well plate format, indicating hours of substrate incubation. Cell lines transfected with a Simplicon™ vector, scaled, frozen and subsequently assayed (6 days, total), demonstrated a similar metabolic profile to those assayed post-transfection. Currently, most commercial high throughput cell based assay systems use gold clean sets, which lack metabolic competence. Here, the MilliporeSigma Simplicon™ Technology is used to create a cell line which expresses and stimulates metabolic activity of five human xenobiotic metabolizing enzymes, and can be scaled to screen thousands of chemical compounds in a 96-well plate format.

**Simplicon Expression of Five CYP450 Enzymes in HepG2 cells and HEK293 cells transfected with Simplicon™ vectors.** 1b: Total RNA was extracted from transfected HEK293 cells and HepG2 cells and quantified by mass spectrometry of the metabolites produced from the substrates testosterone, tolbutamide, chlorzoxazone, phenacetin and buproprion. Relative expression of each enzyme was determined by qPCR measurement. Unlike the HepG2 cell line, to achieve substantial uptake, HEK293 cells were transfected to stably express liver transporters (i.e. OATP1B1 or OATP1B3) while cytochrome P450 metabolites varied with the substrate used, both cell lines show a 10-fold increase of testosterone metabolism over background in a 96-well plate format, indicating hours of substrate incubation. Cell lines transfected with a Simplicon™ vector, scaled, frozen and subsequently assayed (6 days, total), demonstrated a similar metabolic profile to those assayed post-transfection. Currently, most commercial high throughput cell based assay systems use gold clean sets, which lack metabolic competence. Here, the MilliporeSigma Simplicon™ Technology is used to create a cell line which expresses and stimulates metabolic activity of five human xenobiotic metabolizing enzymes, and can be scaled to screen thousands of chemical compounds in a 96-well plate format.

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**Summary**

Previously, MilliporeSigma Simplicon™ Technology was utilized to generate human iPSC cultures from human foreskin fibroblasts via a introduction of four reprogramming factors1. Here, we demonstrate the use of the technology to transiently introduce CYP450 enzymes into HEK293 cells stably expressing a liver transporter, and HEK293 cells transfected with a single polycistronic, self-replicating RNA vector containing five CYP450 enzymes, scaled under puromycin selection, and assayed for metabolic activity of those enzymes in a 96-well plate assay 6 days post-transfection. Three Simplicon™ vectors were created to test the concept. Plan 1 Simplicon™, containing only CYP3A4, was created to determine the transfection and subsequent cell scale-up conditions. The two larger Simplicon™ vectors, containing all five CYP450 enzymes, differing only by the location of the reprogramming factors in the HEK293 cells stably expressing OATP1B1 or OATP1B3. Metabolites were analyzed by mass spectrometry after 3 hour incubation with substrate.

**Figure 1. mRNA expression of five CYP450 enzymes in HepG2 cells and HEK293 cells transfected with Simplicon™ vectors.** 1b: Total RNA was extracted from transfected HEK293 cells and HepG2 cells and quantified by mass spectrometry of the metabolites produced from the substrates testosterone, tolbutamide, chlorzoxazone, phenacetin and buproprion. Relative expression of each enzyme was determined by qPCR measurement. Unlike the HepG2 cell line, to achieve substantial uptake, HEK293 cells were transfected to stably express liver transporters (i.e. OATP1B1 or OATP1B3) while cytochrome P450 metabolites varied with the substrate used, both cell lines show a 10-fold increase of testosterone metabolism over background in a 96-well plate format, indicating hours of substrate incubation. Cell lines transfected with a Simplicon™ vector, scaled, frozen and subsequently assayed (6 days, total), demonstrated a similar metabolic profile to those assayed post-transfection. Currently, most commercial high throughput cell based assay systems use gold clean sets, which lack metabolic competence. Here, the MilliporeSigma Simplicon™ Technology is used to create a cell line which expresses and stimulates metabolic activity of five human xenobiotic metabolizing enzymes, and can be scaled to screen thousands of chemical compounds in a 96-well plate format.

**Figure 2. Requirement of Human Liver Transporters for Functional CYP450 Activity in HEK293 cells.** HEK293 cells stably expressing OATP1B1 or OATP1B3 were transfected with the Plan 3 Simplicon™ and assayed at the progression of phenacetin metabolism. For comparison, a non-CHIR99021 cells stably expressing CYP3A4 and CYP2D6 under a CMV promoter was included. The CHIR99021 vector was transfected HEK293 cells. Transfection of Plan 3 Simplicon™ vectors expressing these transporters were created (data not shown).

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**Figure 3. Activity of four CYP450 enzymes in Plan 1 Transfected HEK293 and HepG2 cells.** HEK293 cells stably expressing OATP1B1 or OATP1B3 cells were transfected with either Plan-1 Simplicon™ vector. Metabolites were analyzed by mass spectrometry after 3 hour incubation with substrate.